

APPLICATION OF
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
TO SOME TECHNICAL ANALYTICAL PROBLEMS

av

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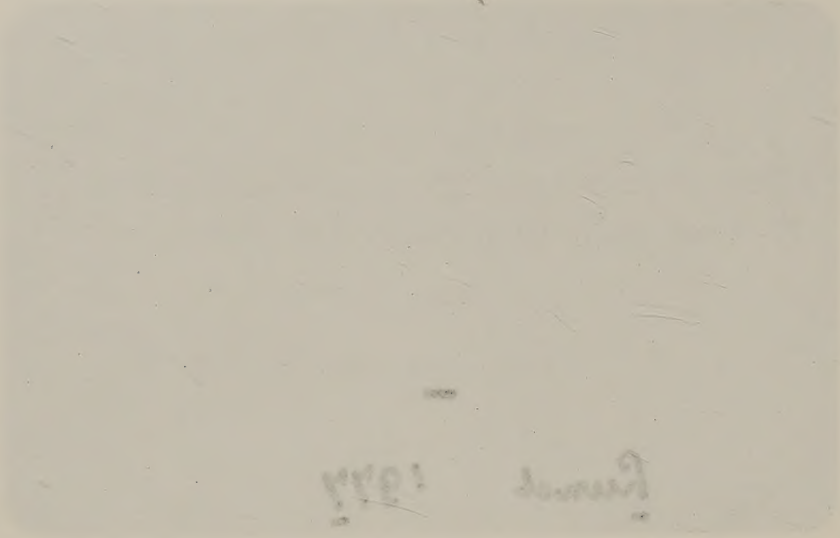
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This review summarizes and discusses results from the following papers (I-V). Some additional results will also be presented.

- I K. Callmer and O. Nilsson. Modification of a Varian Liquid Chromatography UV-Detector for High Sensitivity Measurements. *Chromatographia*, 6 (1973) 517.
- II K. Callmer and L. Davies. Separation and Determination of Vitamin B₁, B₂, B₆ and Nicotinamide in Commercial Vitamin Preparations Using High Performance Cation-Exchange Chromatography. *Chromatographia*, 7 (1974) 644.
- III K. Callmer. Separation and Quantitative Determination of Trimethylolpropane and Pentaerythritols in Industrial Synthesis Solutions by High Performance Liquid Chromatography. *J. Chromatogr.*, 115 (1975) 397.
- IV K. Callmer, L.-E. Edholm and B.E.F. Smith. A Study of Retention Behaviour of Alkylphenols in Straight and Reversed Phase Liquid Chromatography and Application to the Analysis of Complex Phenolic Mixtures in Conjunction with Gas Chromatography. *J. Chromatogr.* In press.
- V L.-E. Edholm and K. Callmer. A Liquid Chromatographic Study of Brominated Alkylphenols and Investigation of Product Formation in the Coulometric Bromination of Some Alkylphenols. *J. Chromatogr.* Submitted for publication.

INTRODUCTION

Although it is generally accepted that modern liquid chromatography began its development in 1964-65, the basis for theoretical and practical treatment dates back to 1941 when the classical work of Martin and Synge was published¹. Their work provided a theoretical background of the chromatographic separation process and the efficiency of the separations achieved was expressed in terms of the theoretical plate height (HETP) by analogy with distillation theory. The paper also introduced a new method of performing separations by partition chromatography - column liquid-liquid chromatography.

One important statement made in this work was that the highest separation efficiency should be obtainable using very small particles. It was not until the early 1970:s that this prediction became fully appreciated and several workers confirmed the advantages of working with particles in the size range below 10 micron^{2,3}.

The development of modern liquid chromatography or "high performance liquid chromatography" (HPLC) as it is preferably called started in the middle 1960:s when the theory of gas chromatography was extended to the field of liquid chromatography. Works of J.C. Giddings, L.R. Snyder, I. Halasz, J.F.K. Huber, B.L. Karger, J.H. Knox, J.J. Kirkland, R.P.W. Scott, and C.G. Horvath have been especially valuable for the understanding and development of HPLC.

BASIC DEFINITIONS AND EQUATIONS

The fundamental retention parameter in LC is the *column capacity ratio* or the *capacity factor*, k' , which is defined as the total moles of solute in the stationary phase (n_s) divided by the total moles of solute in the mobile phase (n_m). The relation between the capacity factor and the distribution constant, K , is given by

$$k' = \frac{n_s}{n_m} = K \cdot \frac{V_s}{V_m} \quad (1)$$

where V_s and V_m are the volumes of the stationary and mobile phases, respectively.

The migration rate v_x of a sample band x in a chromatographic column depends upon the fraction of molecules x in the mobile phase and upon the solvent velocity v

$$v_x = v \cdot \frac{n_m}{n_m + n_s} = \frac{v}{1 + k'} \quad (2)$$

Since $v_x = L/t_R$ and $v = L/t_o$, where L is the column length, t_R the retention time of the band x , and t_o the time required for solvent molecules (or other nonretained compounds) to pass the column, the relation between t_R and t' can be expressed as

$$t_R = t_o (1 + k') \quad (3)$$

Rearrangement of eqn. 3 gives

$$k' = \frac{t_R - t_o}{t_o} \quad (4)$$

Eqn. 4 shows that the capacity factor for the solute can be readily determined from the chromatogram, see Fig. 1. The important column parameter, t_o , can in most cases be determined by the baseline disturbance following sample injection, but in dubious cases it is recommended to inject a nonretained compound.

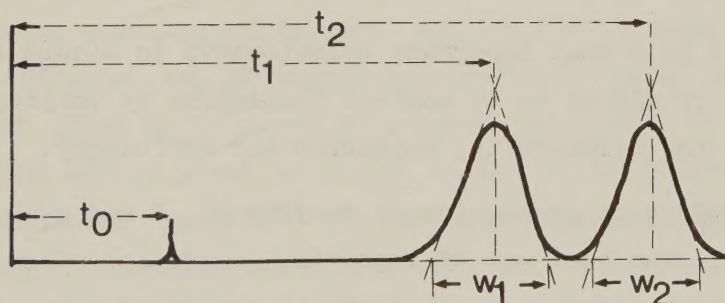


Fig. 1. Chromatogram.

Column efficiency in liquid chromatography is expressed in terms of number of *theoretical plates*, N , or in *plate height*, H (or HETP; $H = L/N$). N is a measure of the band broadening when the band moves along the column and can be obtained from the chromatogram by the following relationship:

$$N = 16 \cdot \left[\frac{t_R}{w} \right]^2 \quad (5)$$

where t_R is the retention time and $w = 4\sigma$ is the band width of the peak, see Fig. 1.

A measure of the separation of two adjacent bands is given by the *resolution*, R_s , and is defined by the distance between the peak maxima divided by the mean value of the band width:

$$R_s = \frac{t_2 - t_1}{1/2(w_1 + w_2)} \quad (6)$$

The relation between resolution (R_s), column efficiency (N), capacity factor (k') and *separation factor* (or *selectivity factor*) $\alpha = k'_2/k'_1$, can be derived from equation 3, 5 and 6. Assuming that $w_1 \approx w_2$ and $N_1 \approx N_2$

$$R_s = \frac{1}{4} \cdot \frac{(\alpha - 1)}{\alpha} \times \frac{k'_2}{1 + k'_2} \times \sqrt{N} \quad (7)$$

$\uparrow$
 selectivity
term

$\uparrow$
 retention
term

$\uparrow$
 efficiency
term

Eqn. 7 is a most important relationship in liquid chromatography since it allows us to control resolution by varying the different terms for selectivity, retention and efficiency.

The effect on resolution by varying k' , N and α is illustrated in Fig. 2. The best way of improving separation often depends on the type of separation problem one is concerned with. The first procedure for improvement is to ensure that the bands are eluted with k' in the optimum range, $1 < k' < 10$. This is obtained by varying the strength of the solvent (*cf.* for instance papers II

and V). Tables of eluotropic series and solubility parameters are useful in this connection^{4,5}.

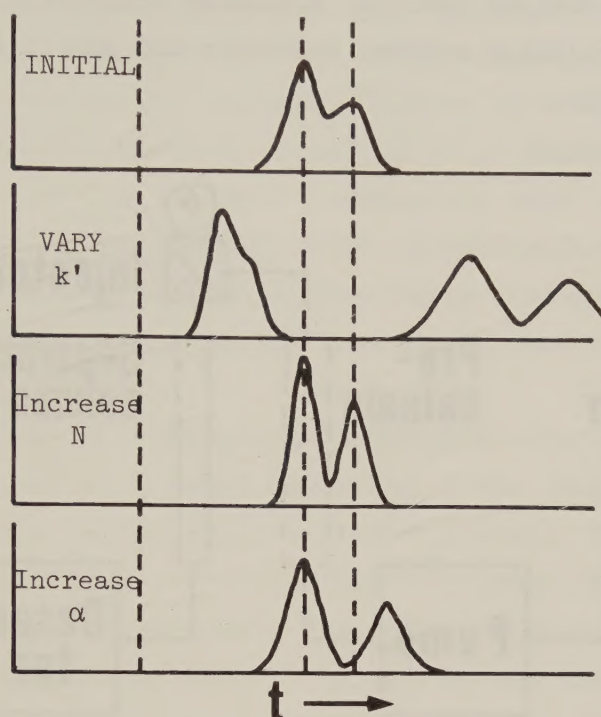


Fig. 2. The effect on resolution between two adjacent bands by varying k' , N and α .

If k' is already within the optimum range and α is not too close to unity, the easiest way of improving resolution is usually to increase N , *e.g.* by increasing the column length, provided that the increased pressure requirement and separation time allow this. The most efficient way of improving resolution is by increasing α . This can often be obtained by a selective choice of the components in the mobile phase utilizing tables of solubility parameters ("Hildebrand scales")^{4,5}. In ion pair chromatography of ionic and ionizable compounds the selectivity can be increased by a proper choice of type and concentration of counter ion^{6,7}.

APPARATUS FOR LIQUID CHROMATOGRAPHY USED

A liquid chromatographic system is schematically shown in Fig. 3. It can be divided into the following sections - solvent delivery system (including solvent reservoir and pump), injector, column and detector.

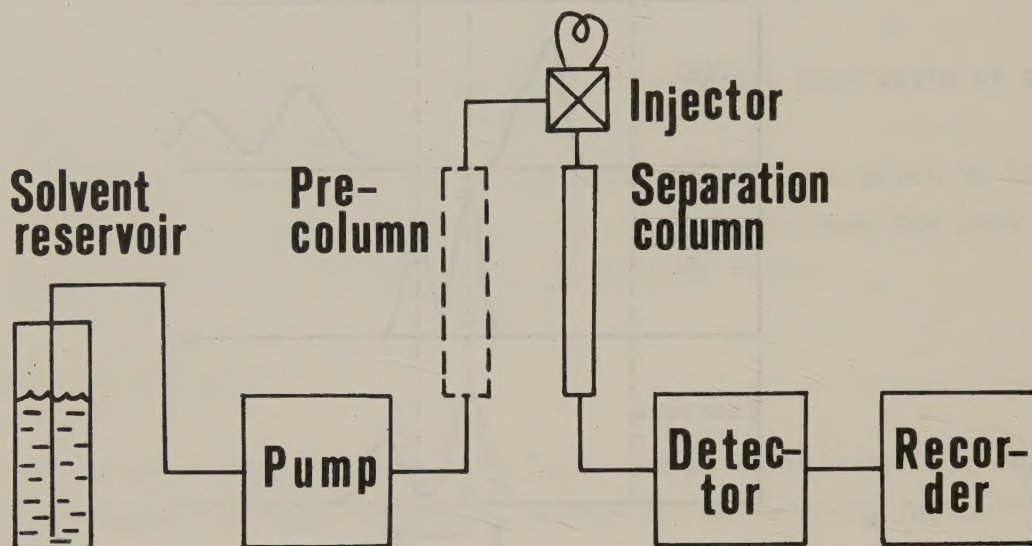


Fig. 3. Equipment for HPLC.

Solvent delivery system

The work described in this thesis has been carried out using two different types of constant flow pumps, namely an *LDC model 711* (Laboratory Data Control) and a *Varian 4100 syringe pump*. The LDC pump consists of a reciprocating Milton Roy Minipump with a flexible bellows as pulse dampening device. Highest operation pressure for the pump is 350 atm although the pulse dampener can be used only up to ca 80 atm. The flow rate can be varied between 20 and 200 ml h⁻¹. A reciprocating pump has the advantage of continuous solvent delivery and it is very well suited for optimizing separation conditions as the solvent can be easily changed. The LDC-pump is convenient to work with and is also relatively cheap. However,

we found that it was not suitable when accurate measurements of retention data were required since the flow-rate varied occasionally and the arrangement for flow adjustment is less satisfactory. Such measurements were therefore preferably performed using the Varian 4100 pump which is of the syringe displacement type (IV, V). It provides a pulseless and very constant flow which can be easily and accurately adjusted ($0 - 200 \text{ ml h}^{-1}$). Maximum operation pressure is 350 atm. The main disadvantage with this type of pump is its finite volume (250 ml) which necessitates refilling of the solvent and makes frequent solvent change inconvenient. However, once the chromatographic conditions are settled it is easy and dependable to work with.

The reliability of retention data obtained with this type of pump has been called in question referring to the compressibility of liquids, which is small but not zero⁸. However, the question seems to be dubious since it concerns the transitory stage, which in practice is always limited in time by pressurizing the liquid, and it is generally a matter of course to wait for steady state conditions before measuring any retention data. For gradient elution work, however, the question is justified and for accurate gradients with these pumps it would be necessary to use constant back pressure valves⁹.

Injection device

There are basically two types of injection systems in liquid chromatography - the *syringe* and the *sample valve*. Syringe injection through a septum usually provides the highest column efficiencies but it can only be used at relatively low pressure. The reproducibility by syringe injection was about 2% at a pressure of 20 atm (II, III) but decreases with increasing pressure (III). Sample valves give slightly lower column efficiency but they provide accurate, reproducible injections at pressures up to 400 atm. This type of injection is recommended for routine analysis. Sample volume is usually changed by varying the loop volume but in some cases it

is possible to inject a sample volume which is smaller than the volume of the loop (V).

Detector

The most widely used detectors in liquid chromatography are the *ultra-violet (UV) detector* and the *refractive index (RI) detector*.

UV-detector. The first paper (I) describes a modification made on one of the early commercial UV-detectors (Varian, 254 nm) in order to improve baseline stability. The bakelite cell house, which in this model was placed immediately above the UV-lamp with poor isolation in between, was gradually heated with serious baseline disturbance as a consequence. It was therefore replaced by a brass-replica equipped with cooling coils. The original stainless steel cells were kept and silver soldered into the brass-replica. The polyethene foils between the quartz lenses and cells were replaced by viton foils. This did indeed improve the tightening between the cells, but the new teflon foils now available are to be preferred because of their chemical inertness. The measures taken improved the baseline stability considerably, and less than 1 ng of biphenyl was readily detected.

In a similar UV-detector, LDC 1285 (280 or 254 nm) which was also used in this work, the cell house is placed beside the UV-lamp with sufficiently good isolation, preventing the cell house from being heated. Although cooling is generally not needed (II-V), this detector can, if necessary, be modified in the same way as described for the Varian detector (I).

A variable wavelength detector (Perkin Elmer LC-55, 190 - 800 nm) was used in some cases (V). It provides good selectivity and stability but the sensitivity at 280 and 254 nm is lower than for the photometers.

RI-detectors are bulk property detectors and are as such principally general detectors. Their main disadvantage is the lack of

sensitivity. In the liquid chromatographic analysis of polyhydric alcohols a refractive index detector of the Fresnel type was used (III). It has a cell volume of 3 μ l and is well suited for work with efficient columns. The sensitivity is in the order of 10^{-3} times less than that of the UV-detectors. For instance, using water as eluent (III) the detection limits for polyhydric alcohols were in the range of 1-5 μ g of injected substance.

COLUMN AND COLUMN PACKING MATERIAL

The separation column is the most important part of the liquid chromatographic system and much research has been devoted to optimizing column design, packing material and packing techniques. The column is usually of stainless steel with the dimensions 100-300 mm in length, 6.35 mm (1/4 inch) O.D. and 3-5 mm I.D. Such columns with microparticulate packing material (about 10 μ m or less) provide a good compromise between analytical and semi-preparative separations (sample size up to several milligrams of solute). If pellicular packing (40-50 μ m) is used the column length is usually 1 m. While microparticulate packings with high resolving power are to be used to separate complex mixtures of solutes of similar structure (IV, V), pellicular packings are preferably used in the case of less complex mixtures of wide polarity range (II).

Silica is the most widely used packing material for adsorption chromatography (LSC) and as support material in liquid-liquid chromatography (LLC) and in bonded phase chromatography (BPC). It is available with well defined properties (surface area, pore diameter and pore volume) in narrow-ranged particle sizes of irregular or spherical shape.

In this work LSC was used for the separation of trimethylolpropane (TMP) in a technical synthesis solution using pellicular Corasil II as support (III). Although LSC is most frequently used for chromatography of lipophilic compounds it was here successfully applied to the hydrophilic TMP, using water as eluent. In this investigation

a column, slurry-packed by a balanced density method² with Merckosorb silica micro-particles ($10\text{ }\mu\text{m} \pm 2$, Merck) was also utilized. It gave HETP-values around 0.1 mm.

In spite of its adsorption properties, silica is the most frequently used support material in LLC, including high efficiency ion pair chromatography^{7,12}. In the study of retention behaviour of alkylphenols on nitrile phases, the conventionally coated liquid phases were applied on Poracil C ($37-75\text{ }\mu\text{m}$) by the evaporation technique and then drypacked (IV). These columns provided HETP-values of about 2 mm. If higher efficiency is required silica micro-particles can, of course, be utilized as support material and in that case the stationary phase is applied by an *in situ* coating technique¹¹⁻¹³.

Silica is also the general support for manufacturing *chemically bonded phases*¹⁴⁻¹⁷, which are available with a wide range of functionalities like NH_2 , CN, Ph, ion-exchanging groups and C_2-C_{18} hydrocarbons. Of these the hydrocarbon phases, and especially the C_{18} phase, are the most frequently used. Recently chemically bonded silica packings have been prepared by bulk modification, whereby the organic functional groups become a constituent of the bulk phase as well as the surface²³.

It is estimated that more than 60% of the HPLC separations today are performed on chemically bonded alkylsilane phases¹⁸. They are preferably used for the separation of polar compounds with water-alcohol or water-acetonitrile eluents (IV, V). However, neat aqueous solutions can also be used¹⁸ (III). In different types of ion pair chromatography suitable counter ions are added to the mobile phase¹⁹⁻²¹. In fact, many separations which were previously performed by ion exchange chromatography can with advantage be carried out on alkylsilane phases²². Bonded alkylsilane phases are also used as support material in reversed phase ion pair partition chromatography⁷.

A chemically bonded, pre-packed nitrile phase, Cyano Sil-X-I ($13\text{ }\mu\text{m} \pm 5$, Perkin-Elmer) was used in addition to the conventionally coated nitrile phases in the studies of retention behaviour of alkylphenols and brominated alkylphenols (IV, V). HETP-values around 0.5 mm were

obtained. Likewise, a prepacked octadecylsilane column, μ Bondapack C₁₈ ($10\ \mu\text{m} \pm 2$, Waters) was used in these investigations. It provided HETP-values of 0.05 - 0.1 mm. This column was also utilized for the determination of polyols in a technical synthesis solution (III).

Ion-exchangers for HPLC are either pellicular²⁴ or consist of silica micro-particles with bonded species containing basic or acidic functional groups¹⁵. Columns that were drypacked with a pellicular ion-exchanger, HS Pellionex SCX ($40 - 50\ \mu\text{m}$, Whatman) were used for the determination of water soluble vitamins (II). HETP-values for these columns were 1 - 2 mm.

RETENTION IN BONDED PHASE CHROMATOGRAPHY (BPC)

The retention mechanism in bonded phase chromatography - and especially on nonpolar phases - has been discussed in more or less qualitative terms by several authors^{20-25,27} (III-V). However, it is not until recently that the problem has been the subject of more detailed investigations^{18,28}.

Retention in reversed phase BPC is controlled by varying the concentration of an organic modifier and its influence on retention can be directly correlated to changes in surface tension¹⁸ in analogy with the case in conventional LLC and extraction²⁹.

Westerlund *et al.*²⁰ showed that partition effects between adsorbed modifier and mobile phase contribute to the retention of ion pairs on an alkylsilane column with methanol-water as eluent. Thus, partition occurs between the mobile phase and a spontaneously adsorbed layer of methanol (0.1 ml/g) on the support. It has also been shown that individual compounds of a similar type are separated on the basis of their relative solubility in the mobile phase²⁶ (III).

Dispersive interactions are likely to be involved in the retention mechanism of solutes containing nonpolar substituents, *e.g.* interaction between alkyl groups in a molecule and the hydrocarbonaceous ligands at the surface of the stationary phase^{25,27,30} (III, IV). This is exemplified by Fig. 4 wherein $\log k'$ is plotted *vs.* carbon number

(C_n) for some aliphatic alcohols.

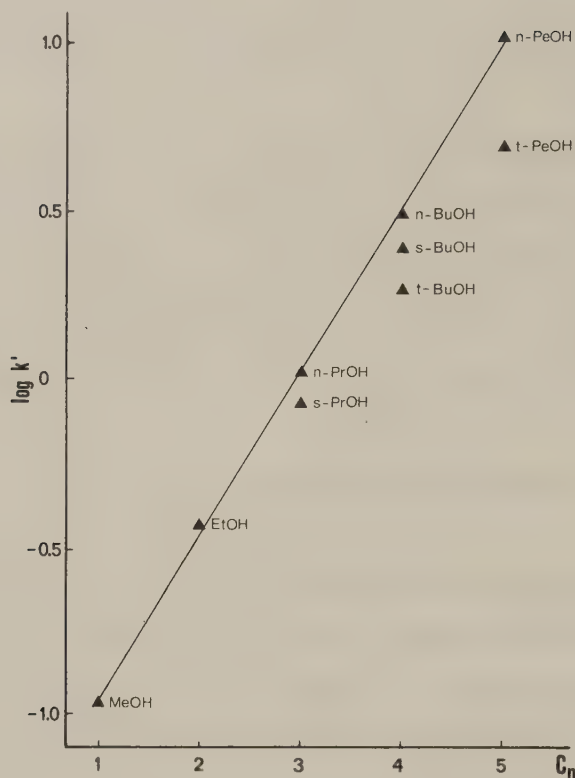


Fig. 4. Log k' vs. carbon number for aliphatic alcohols. Column: μ Bondapak C_{18} . Eluent: methanol-water, 5:95 (v/v) (IV).

The points for normal alcohols lie on a straight line, while those for secondary and tertiary alcohols lie below the line. The same relation between retention and degree of branching of alkyl groups was observed for *para*-substituted alkylphenols (IV), while for *ortho*-substituted this effect is cancelled by the steric hindrance of the hydroxyl, which *inter alia* influences the solubility in the mobile phase.

Recently Horvath *et al.*¹⁸ and Karger *et al.*²⁸ have explained the retention in reversed phase BPC in terms of the hydrophobic effect and this seems to provide a sound basis for the practical and theoretical treatment of such chromatographic data. While hydrophobic effects are prevalent in a neat aqueous eluent, Horvath suggested a "solvophobic effect" to be responsible for retention of solutes in mixed solvent systems. This effect involves surface tension (the

dominating parameter, see also²⁹), solubility parameters and hydrocarbonaceous surface area of the solute. This is largely in agreement with qualitative results from other workers²⁵⁻²⁷ (III, IV) including results obtained on other types of non-polar stationary phases, such as graphite³⁰.

The retention mechanism in straight phase BPC is often easier to interpret since it involves specific interactions between the solute and the functional groups of the ligands. For instance, in the retention of alkylphenols on chemically bonded nitrile phase (IV) the retention mechanism is likely to involve hydrogen bonding between the hydrogen atom of the phenolic hydroxyl group and the nitrogen in the cyano group, see p. 19.

COLUMN STABILITY

Long range stability of the column is of decisive importance in the choice of liquid chromatographic system for routine analyses. Accordingly this property was tested for the pellicular ion exchange resin used in the determination of water-soluble vitamins (II) and for the Corasil II and μ Bondapak C₁₈ columns used in the analyses of polyhydric alcohols (III). No change in performance of the pellicular ion exchange column could be observed after 4 months of continuous elution with (predominantly) dilute nitric acid. However, subsequent investigations have shown that prolonged elution with phosphate buffer (pH = 8) caused serious changes in column performance³¹, which was attributed to corrosion of the non-covered parts of the glass beads, as indicated by means of scanning electron micrographs, see Fig. 5.

The Corasil II column, that was used in the determination of trimethylolpropane with water as eluent, showed no sign of column change after 5 months and more than 500 injections of dilute technical synthesis solutions (III). Column changes could not be observed for the μ Bondapak C₁₈ column either using water as eluent (III), and in subsequent analyses one and the same column was successfully used for more than 5 months during which more than 1200 analyses³² were carried out. The long range stability is improved by occasionally

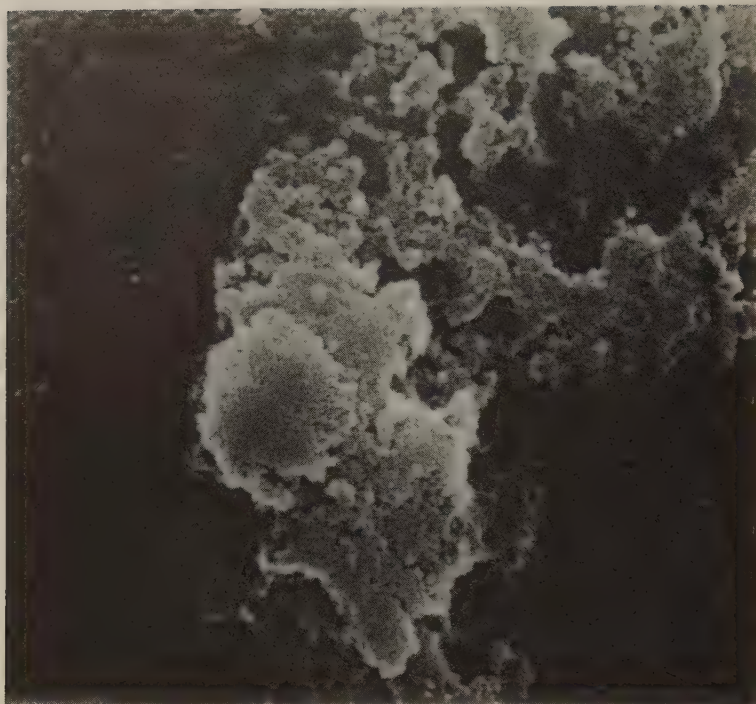


Fig. 5. Scanning electron micrograph of a HS Pellionex SCX particle, which has been exposed to phosphate buffer (0.1 mole l^{-1} , $\text{pH} = 8$) during 2 months. Magnification: 5000 x.

eluting the column with methanol-water, 50:50 (v/v).

The chemically bonded nitrile phase showed no tendencies to deteriorate during the study of the retention behaviour of alkylphenols (IV). However, in the liquid chromatographic investigation of the products at coulometric bromination (V) a 10% decrease in the retention of the reference substance (2,6-dimethylphenol) was noted. This change in column performance is probably due to the fact that the sample from the bromination reaction, which was injected directly onto the column, contained 60% of acetic acid. Irreversible reactions may occur between the acetic acid and active sites on the support, *i.e.* cyano groups and/or remaining silanol groups.

One of the main arguments for the chemically bonded phases, when these were first introduced, was the high column stability obtained, due to the fact that the stationary phase cannot be eluted from the support. Indeed, this is true but it does unfortunately not exclude

other column changes, such as irreversible reactions with contaminated samples. This is, for instance, a problem which practically always arises when working with biological samples. Referring to long range column stability it would, in fact, be advantageous to use conventionally coated stationary phases since they "protect" the support material and can, if required, be washed out from the column and replaced with new phase.

However, in practice it is more convenient to work with chemically bonded phases, since no pre-equilibration and thermostating of the mobile phase is needed and the composition of the eluent can be varied unimpededly when optimizing a separation.

In order to minimize column changes, when working with chemically bonded phases, certain precautions can be taken, such as injection of "clean" samples, use of pre-column, flushing or back-flushing with a suitable eluent etc.

CHOICE OF LIQUID CHROMATOGRAPHIC CONDITIONS

Water soluble vitamins (II)

The water soluble vitamins B_1 (thiamine), B_2 (riboflavin), $B_2\text{-PO}_4$ (riboflavinphosphate), B_6 (pyridoxine) and nicotinamide are bases, principally due to the presence of aromatic nitrogen. They are consequently cat-ions at sufficiently low pH and are retained by cat-ion exchangers. The difference in molecular structure between the vitamins are great enough to allow their separation on a *pellicular ion exchanger* (HS Pellionex SCX). The retention is controlled by means of pH and ionic strength of the eluent. For the separation of B_2 (or $B_2\text{-PO}_4$), B_6 and nicotinamide dilute nitric acid ($0.025 - 0.05$ mole l^{-1}) was used as eluent, while the elution of B_1 required pH about 8 in a phosphate buffer concentration of 0.1 mole l^{-1} . The long range stability of the column was good with the conditions used and no change in column performance was observed during the course of the work. This was also indicated by comparing scanning electron micrographs of a sample of "fresh" support with those of a sample taken from a column which had been in continuous use for

4 months. No visible difference in surface characteristics could be observed between the two samples. However, this result refers to the long range stability of a resin in dilute nitric acid, pH = 2, which had only occasionally and for short periods been eluted with 0.1 M phosphate buffer, pH = 8. When the resin was exposed to the pH 8 eluent for a longer period of time, serious decrease in column performance was observed. Scanning electron micrographs revealed that the non-covered areas of the glass beads were seriously corroded, see Fig. 5. The result is attributed to the high pH of the solution and according to the manufacturer the upper limit has by now been lowered from the originally recommended 13 (!) to 7.5.

The short exposures to pH 8 eluent that occurred in the elution of vitamin B₁ (II) might have etched the glass beads enough to produce active sites, which caused the observed non-linear isotherm for small sample sizes.

An attempt to chromatograph water soluble vitamins on a silica based *micro-particulate ion exchanger* (Partisil SCX) resulted in severely tailed peaks which was probably due to rest silanol groups.

Vitamins B₂, B₆ and nicotinamide can also be separated by means of *reversed phase ion pair chromatography*¹⁷. The influence of pH on k' for B₁, B₂, B₂-PO₄ and nicotinamide on a μ Bondapak C₁₈ column is shown in Fig. 6³¹.

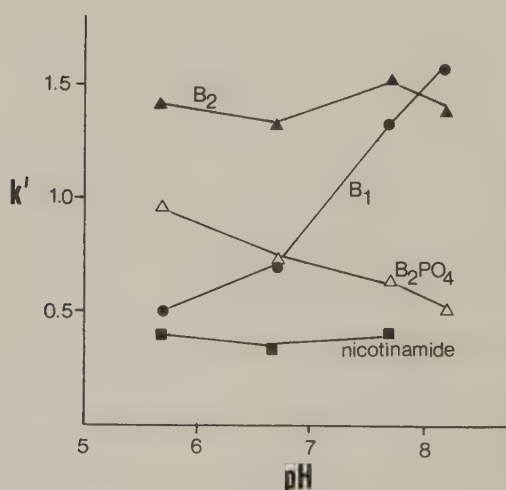


Fig. 6. k' for vitamin B₁, B₂, B₂-PO₄ and nicotinamide in reversed-phase BPC as a function of pH. Column: μ Bondapak C₁₈; Eluent: 0.1 mole l⁻¹ ammonium phosphate buffer-methanol, 65:35 (v/v).

As can be seen it should be possible to separate vitamin B₁ from B₂ and B₂-PO₄ using the conditions in Fig. 6, at pH 7.5.

In a study of the degradation of vitamin B₁³¹, μ Bondapak C₁₈ was likewise found to separate B₁ from many of its decomposition products, see Fig. 7.

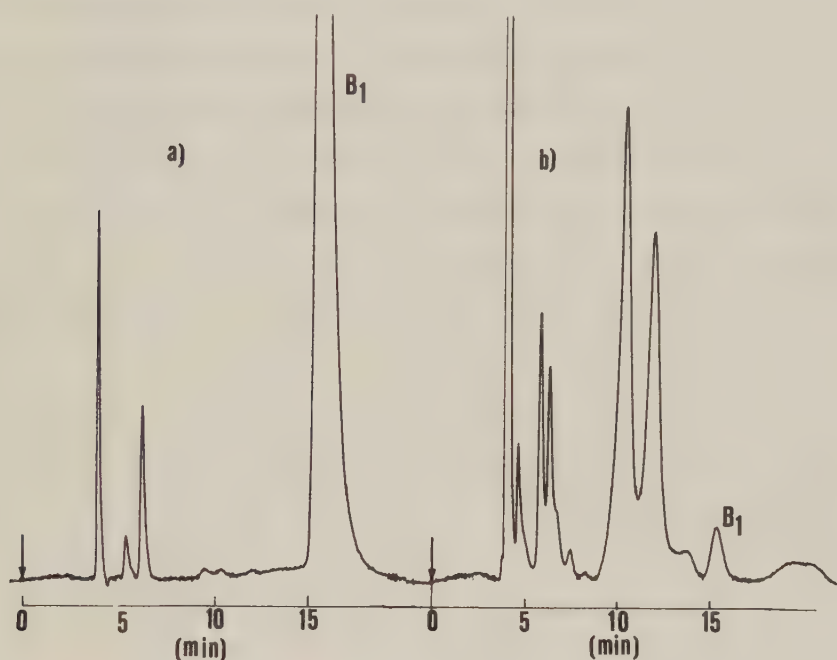


Fig. 7. Chromatogram showing the separation of vitamin B₁ and some of its degradation products. Column: μ Bondapak C₁₈. Eluent: 1% aqueous (NH₄)₂CO₃ solution-methanol, 65:35 (v/v), pH ~ 9.5. Flow-rate: 40 ml h⁻¹. Detection wavelength 230 nm.

Fig. 7 a) B₁ from a pH 4 solution.

Fig. 7 b) "B₁" from a pH 10 solution (ca 3 h after preparation).

However, as pH in this solution is above 9, it should not be left in contact with the column packing for a longer period of time since the Si-O-Si bonds can be attacked at this pH.

Polyhydric alcohols (III)

The polyhydric alcohols trimethylolpropane (TMP) and monopentaerythritol (MPE) are important raw materials for the manufacture

of plastics. They are strongly polar compounds and gas chromatographic analysis requires tedious and complicated sample treatment.

Adequate separations of the components in a technical synthesis solution of TMP was achieved on four different columns using water as the mobile phase. The retention of TMP on the columns increased in the order *pellicular silica particles* ($k' = 0.3$) < *silica microparticles* ($k' = 0.4$) < *cyano bonded phase* ($k' = 0.7$) < *octadecylsilane (ODS) bonded phase*, μ Bondapak C₁₈ ($k' = 2.2$). The high retention value of TMP on the ODS-column is supposed to be caused by dispersive interactions between the hydrocarbonaceous ligands in the stationary phase and the non-polar part of the TMP-molecule. The components in technical synthesis solutions of MPE were separated on a μ Bondapak C₁₈ column using water as eluent.

The HPLC methods for determination of TMP and MPE afford considerable advantages compared to GC-methods. Thus, after changing from GC to LC-analyses³² in a polyol-manufacturing industry the number of analyses could be increased from about 2 to about 25 per day. This improvement made it possible to conveniently follow the synthesis and optimize the conditions.

It is of interest to note that in spite of the neat aqueous mobile phase most of the compounds were eluted as peaks with good symmetry. Similar results have been reported by other workers¹⁸ but it is not always the case when working with alkylsilane bonded phases^{7,28}. The difference in performance in this respect can be explained by the fact that the surface coverage of alkylsilane bonded phases varies between supports from different manufacturers and even between different batches. Remaining silanol groups facilitate wetting by the neat aqueous eluent thus minimizing mass transfer resistance¹⁸. As a consequence high column efficiency in this type of LC-system would indicate the presence of silanol groups.

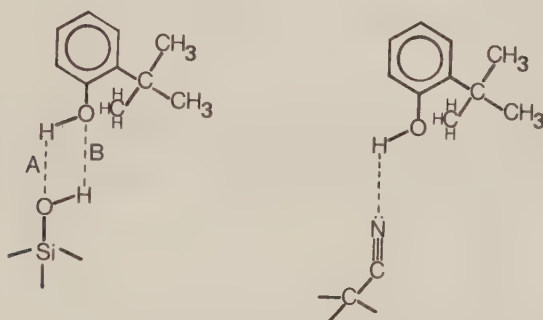
Alkylphenols (IV)

Alkylphenols are important raw materials for the synthesis of a wide range of organic compounds, pharmaceuticals, plasticizers, antioxi-

dants, etc. They constitute an essential part of the components in coal tar and certain petroleum fractions. On that account, their qualitative and quantitative analysis is of considerable interest.

The retention behaviour of a series of alkylphenols was investigated using three straight phase systems (*nitrile phases*) and one reversed phase system (*ODS-bonded phase*). On the nitrile phases the alkylphenols are eluted in the order: di-, mono- and non-*ortho*-substituted phenols which is primarily due to decreased steric hindrance to hydrogen bonding in the same order.

The reversed elution order of 2,6-dimethyl- and 2-*tert.*-butylphenol on silica and nitrile phases, respectively, can be explained by the difference in hydrogen bond interactions:



The surface silanol groups are capable of forming hydrogen bonds with both the oxygen and the hydrogen atom of the phenolic hydroxyl group although the former interaction (marked B in the figure) is the one generally considered in the literature. The cyano group, on the other hand, is only capable of forming a hydrogen bond to the hydrogen atom of the phenolic hydroxyl. As indicated by the figure, a bulky *ortho* substituent, *e.g.* *tert.*-butyl, should interfere to a greater extent with the dominating B-type hydrogen bonding to the silanol group than with the hydrogen bonding to the cyano group. Accordingly, 2-*tert.*-butylphenol is eluted earlier than 2,6-dimethylphenol on silica, while the opposite is valid on a nitrile phase. Low surface coverage on a chemically bonded phase (Cyano Sil-X-I), as compared to highly loaded LLC nitrile columns, increases the contri-

bution to retention by adsorption to remaining silanol groups, making the elution order more similar to that on silica.

In reversed phase chromatography it is primarily the number of alkyl carbon atoms that determines the retention, although there is a certain positional influence. Two-phase plots, *i.e.* $\log k'$ on reversed phase *vs.* $\log k'$ on straight phase give three well separated straight lines representing di-, mono- and non-*ortho*-substituted phenols, see Fig. 8, which makes it possible to distinguish between the three types of alkylphenols.

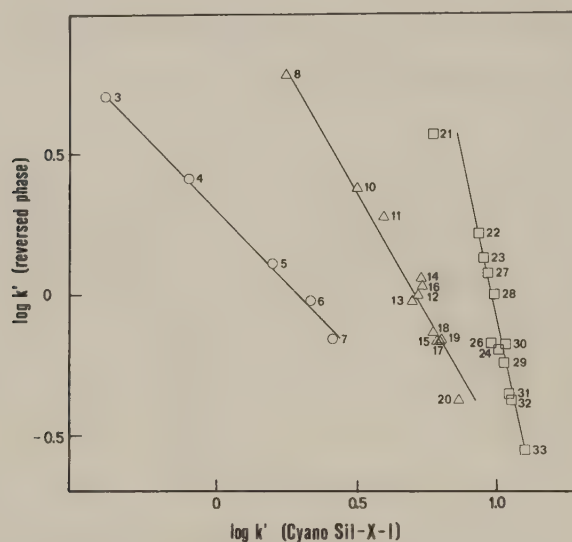


Fig. 8. Two-phase plot for alkylphenols. $\circ$ di-*ortho*-, Δ mono-*ortho*-, $\square$ non-*ortho*-substituted phenols.

Brominated alkylphenols (V)

The fifth paper describes the application of HPLC to the analysis of product formation at coulometric bromination of alkylphenols. The combination of HPLC and coulometric bromination by the method described by Kinberger *et al.*³³ offers a unique possibility for the study of retention characteristics of brominated phenols since a practically unlimited number of *ortho*- and *para*-brominated phenols can be readily synthesized. The main bromination reaction is exchange of hydrogen for bromine in the free *ortho* and *para* posi-

tions and the coulometric method generally yields one unambiguous product for the phenols investigated. The study concerns three types of alkylphenols: 4-alkylphenols and 2,4- and 2,6-dialkylphenols. The brominated products are chromatographed by *straight* and *reversed BPC*.

For all phenols investigated the introduction of bromine results in an increase of the retention on the reversed phase system independently of whether the substitution takes place in an *ortho* or *para* position. For each group of phenols there is a linear relationship in the reversed phase system between the capacity factor for the brominated (k'_2) and non-brominated (k'_1) phenols, *i.e.*

$$k'_2 = m \cdot k'_1 + b \quad (8)$$

The linear correlation to eqn. 8 for 2,6-dialkylphenols, 4-alkylphenols and 2-bromo-4-alkylphenols is summarized in Table I.

Table I. Linear correlation (r) between k' -values of brominated (k'_2) and non-brominated (k'_1) phenols. Column: μ Bondapak² C₁₈. Eluent: ethanol/water 60:40, v/v.

Compounds	$k'_2 = mk'_1 + b$			
	m	b	r	n
2,6-Dialkylphenols	1.30	0.43	0.9990	7
4-Alkylphenols*	1.22	0.19	0.9998	7
2-Bromo-4-alkylphenols*	1.43	0.15	0.9997	7

*Values for 4-cyclohexylphenol and 2-bromo-4-cyclohexylphenol omitted.

It follows from eqn. 8 that the separation factor, α , is a hyperbolic function of k'_1

$$\alpha = \frac{k'_2}{k'_1} = m + \frac{b}{k'_1} \quad (9)$$

which has the asymptot $\lim_{k'_1 \rightarrow \infty} \alpha = m$. Thus it can be expected that for

alkylphenols with large k' -values (high alkyl carbon number) the separation factor, α , between the brominated and non-brominated phenol approaches the asymptotic value of m (see Table I). Plots of α vs. k'_1 for the phenols in Table I are shown in Fig. 9.

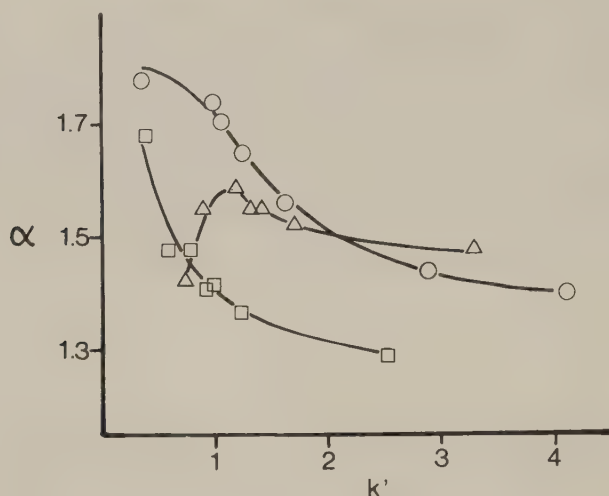


Fig. 9. Separation factor, α , vs. k'_1 , for brominated/non-brominated alkylphenols on μ Bondapak C_{18} . Eluent: ethanol/water 60:40. $\circ$ 2,6-Dialkylphenols; $\square$ 4-Alkylphenols; $\triangle$ 2-Bromo-4-alkylphenol.

Deviation from linearity was observed when k'_2 was plotted vs. k'_1 for 2,6-dimethylphenol, 2-bromo-4-methyl- and 2-bromo-4-ethylphenol (V) and this is also reflected in the deviation from the hyperbolic function in Fig. 9 for these phenols.

The α -values for the phenols with the largest alkyl carbon number (or the highest k' -values) are for the different groups: $\alpha = 1.40$ for 2,6-di-*tert.*-butylphenol, 1.29 for 4-octylphenol and 1.48 for 2-bromo-4-octylphenol. These values compare favourably with the slopes (m) in Table I.

In the straight phase LC-system introduction of bromine into the *ortho* position of 2,4-dialkyl- and 4-alkylphenols results in a marked decrease in retention due to decreased strength in the hydrogen bonding to the stationary phase caused by steric hindrance of the phenolic hydroxyl group. Introduction of bro-

mine into the *para* position of 2,6-dialkylphenols causes an increase in retention with a linear correlation of 0.999 between k' for brominated and non-brominated phenols.

The combination of coulometric bromination and liquid chromatography can be used for separation of isomeric alkylphenols, which are difficult to separate as such by chromatographic methods, *e.g.* 3- and 4-methylphenol.

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